



(19)

Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) EP 0 931 835 A2

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:
28.07.1999 Bulletin 1999/30

(51) Int Cl. 6: C12N 15/55, C12N 15/54,
C12N 9/22

(21) Application number: 98309629.8

(22) Date of filing: 24.11.1998

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: 24.11.1997 US 976703

(71) Applicant: NEW ENGLAND BIOLABS, INC.
Beverly Massachusetts 01915 (US)

(72) Inventors:
• Chang, Zhiyuh
New Rochelle, NY 10805 (US)
• Morgan, Richard D.
Middleton, MA 01949 (US)

(74) Representative: Davies, Jonathan Mark
Reddick & Gross
16 Theobalds Road
London WC1X 8PL (GB)

(54) Method for cloning and producing the *Pmel* restriction endonuclease

(57) The present invention relates to recombinant DNA which encodes the *Pmel* restriction endonuclease

and modification methylase, and production of *Pmel* restriction endonuclease from the recombinant DNA.

EP 0 931 835 A2

Description**BACKGROUND OF THE INVENTION**

5 [0001] The present invention relates identification of the *Pmel* restriction endonuclease, obtainable from *Pseudomonas mendocina* (NEB#698) and to the recombinant DNA which encodes the *Pmel* restriction endonuclease, and the production of *Pmel* restriction endonuclease from the recombinant DNA.

10 [0002] Type II restriction endonucleases are a class of enzymes that occur naturally in bacteria. When they are purified away from other bacterial components, restriction endonucleases can be used in the laboratory to cleave DNA molecules into precise fragments for molecular cloning and gene characterization.

15 [0003] Restriction endonucleases act by recognizing and binding to particular sequences of nucleotides (the 'recognition sequence') along the DNA molecule. Once bound, they cleave the molecule within, or to one side of, the recognition sequence. Different restriction endonucleases have affinity for different recognition sequences. Over one hundred and eighty restriction endonucleases with unique specificities have been identified among the many hundreds of bacterial species that have been examined to date.

20 [0004] Bacteria tend to possess at most, only a small number of restriction endonucleases per species. The endonucleases typically are named according to the bacteria from which they are derived. Thus, the species *Deinococcus radiophilus* for example, synthesizes three different restriction endonucleases, named *DraI*, *DraII* and *DraIII*. These enzymes recognize and cleave the sequences TTTAAA, PuGGNCCPy and CACNNNGTG, respectively. *Escherichia coli* RY13, on the other hand, synthesizes only one enzyme, *EcoRI*, which recognizes the sequence GAATTC.

25 [0005] It is thought that in nature, restriction endonucleases play a protective role in the welfare of the bacterial cell. They enable bacteria to resist infection by foreign DNA molecules like viruses and plasmids that would otherwise destroy or parasitize them. They impart resistance by cleaving invading foreign DNA molecule each time that the recognition sequence occurs. The cleavage that takes place disables many of the infecting genes and renders the DNA susceptible to further degradation by non-specific nucleases.

30 [0006] A second component of bacterial protective systems are the modification methylases. These enzymes are complementary to restriction endonucleases and they provide the means by which bacteria are able to protect their own DNA and distinguish it from foreign, infecting DNA. Modification methylases recognize and bind to the same recognition sequence as the corresponding restriction endonuclease, but instead of cleaving the DNA, they chemically modify one or other of the nucleotides within the sequence by the addition of a methyl group. Following methylation, the recognition sequence is no longer cleaved by the restriction endonuclease. The DNA of a bacterial cell is always modified by virtue of the activity of its modification methylase. It is therefore insensitive to the presence of the endogenous restriction endonuclease. It is only unmodified, and therefore identifiably foreign DNA, that is sensitive to restriction endonuclease recognition and cleavage.

35 [0007] With the advent of genetic engineering technology, it is now possible to clone genes and to produce the proteins and enzymes that they encode in greater quantities than are obtainable by conventional purification techniques. The key to isolating clones of restriction endonuclease genes is to develop a simple and reliable method to identify such clones within complex 'libraries', i.e. populations of clones derived by 'shotgun' procedures, when they occur at frequencies as low as 10⁻³ to 10⁻⁴. Preferably, the method should be selective, such that the unwanted majority of clones are destroyed while the desirable rare clones survive.

40 [0008] Type II restriction-modification systems are being cloned with increasing frequency. The first cloned systems used bacteriophage infection as a means of identifying or selecting restriction endonuclease clones (*EcoRI*: Kosykh et al., *Molec. Genet.* 178:717-719 (1980); *HhaI*: Mann et al., *Gene* 3:97-112 (1978); *PstI*: Walder et al., *Proc. Natl. Acad. Sci.* 78:1503-1507 (1981)). Since the presence of restriction-modification systems in bacteria enable them to resist infection by bacteriophages, cells that carry cloned restriction-modification genes can, in principle, be selectively isolated as survivors from libraries that have been exposed to phage. This method has been found, however, to have only limited value. Specifically, it has been found that cloned restriction-modification genes do not always manifest sufficient phage resistance to confer selective survival.

45 [0009] Another cloning approach involves transferring systems initially characterized as plasmid-borne into *E. coli* cloning plasmids (*EcoRV*: Bougueret et al., *Nucl. Acid. Res.* 12:3659-3676 (1984); *PaeR7*: Gingeras and Brooks, *Proc. Natl. Acad. Sci. USA* 80:402-406 (1983); Theriault and Roy, *Gene* 19:355-359 (1982); *PvuII*: Blumenthal et al., *J. Bacteriol.* 164:501-509 (1985)).

50 [0010] A third approach which is being used to clone a growing number of systems, involves selection for an active methylase gene (U.S. Patent No. 5,200,933 and *BsuRI*: Kiss et al., *Nucl. Acid. Res.* 13:6403-6421 (1985)). Since restriction and modification genes are often closely linked, both genes can often be cloned simultaneously. This selection does not always yield a complete restriction system however, but instead yields only the methylase gene (*BspRI*: Szomolanyi et al., *Gene* 10:219-225 (1980); *BcnI*: Janulaitis et al., *Gene* 20:197-204 (1982); *BsuRI*: Kiss and Baldauf, *Gene* 21:111-119 (1983); and *MspI*: Walder et al., *J. Biol. Chem.* 258: 235-1241 (1983)).

[0011] Another method for cloning methylase and endonuclease genes is based on a colorimetric assay for DNA damage (U.S. Patent No. 5,498,535). When screening for a methylase, the plasmid library is transformed into the host *E.coli* strain such as AP1-200. The expression of a methylase will induce the SOS response in an *E.coli* strain which is *McrA*⁺, *McrBC*⁺, or *Mrr*⁺. The AP1-200 strain is temperature sensitive for the *Mcr* and *Mrr* systems and includes a *lac-Z* gene fused to the damage inducible *dinD* locus of *E.coli*. The detection of recombinant plasmids encoding a methylase or endonuclease gene is based on induction at the restrictive temperature of the *lacZ* gene. Transformants encoding methylase genes are detected on -LB agar plates containing X-gal as blue colonies. (Piekarowicz, et.al., *Nucleic Acids Res.* 19:1831-1835 (1991) and Piekarowicz, et.al. *J. Bacteriology* 173:150-155 (1991)). Likewise, the *E.coli* strain ER1992 contains a *dinD1-Lac Z* fusion but is lacking the methylation dependent restriction systems *McrA*, 5 *McrBC* and *Mrr*. In this system (called the "endo-blue" method), the endonuclease gene can be detected in the absence of its cognate methylase when the endonuclease damages the host cell DNA, inducing the SOS response. The SOS-induced cells form deep blue colonies on LB agar plates supplemented with X-gal. (Xu et.al. *Nucleic Acids Res.* 22: 10 2399-2403 (1994)).

[0012] Sometimes the straight-forward methylase selection method fails to yield a methylase (and/or endonuclease) 15 clone due to various obstacles. See, e.g., Lunnen, et.al., *Gene*, 74(1):25-32 (1988). One potential obstacle to cloning restriction-modification genes lies in trying to introduce the endonuclease gene into a host not already protected by modification. If the methylase gene and endonuclease gene are introduced together as a single clone, the methylase must protectively modify the host DNA before the endonuclease has the opportunity to cleave it. On occasion, therefore, it might only be possible to clone the genes sequentially, methylase first then endonuclease.

[0013] Another obstacle to cloning restriction-modification systems lies in the discovery that some strains of *E.coli* react adversely to cytosine or adenine modification; they possess systems that destroy DNA containing methylated cytosine (Raleigh and Wilson, *Proc. Natl. Acad. Sci., USA* 83:9070-9074 (1986)) or methylated adenine (Heitman and Model, *J. Bact.* 196:3243-3250 (1987); Raleigh, Trimarchi, and Revel, *Genetics*, 122:279-296 (1989) Waite-Rees, et al., *J. Bacteriology*, 173:5207-5219 (1991)). Cytosine-specific or adenine-specific methylase genes cannot be cloned 20 easily into those strains, either on their own, or together with their corresponding endonuclease genes. To avoid this problem, it is necessary to use mutant strains of *E.coli* (*McrA*⁺ and *McrB*⁺ or *Mrr*⁺) in which these systems are defective.

[0014] A third potential difficulty is that some restriction endonuclease and methylase genes may not express in *E.coli* due to differences in the transcription machinery of the source organism and *E.coli*, such as differences in promoter 25 and ribosome binding sites. The methylase selection technique requires that the methylase express well enough in *E.coli* to fully protect at least some of the plasmids carrying the gene.

[0015] Because purified restriction endonucleases, and to a lesser extent, modification methylases, are useful tools 30 for characterizing genes in the laboratory, there is a commercial incentive to obtain bacterial strains through recombinant DNA techniques that synthesize these enzymes in abundance. Such strains would be useful because they would simplify the task of purification as well as providing the means for production in commercially useful amounts.

SUMMARY OF THE INVENTION

[0016] The present invention relates to recombinant DNA encoding the genes for the *Pmel* restriction endonuclease 40 (NEB #560), an enzyme which recognizes the DNA sequence 5' GTTTAAC 3' and cleaves at the middle of the recognition sequence between the third T and first A residue to produce blunt ends, as well as related methods for the production of this enzyme from the recombinant DNA. This invention also relates to a transformed host which expresses the restriction endonuclease *Pmel*. *Pmel* restriction endonuclease produced in accordance with the present invention is substantially pure and free of contaminants normally found in restriction endonuclease preparations made by conventional techniques.

DESCRIPTION OF THE DRAWINGS

[0017] Figure 1 is a photograph of an agarose gel demonstrating *Pmel* restriction endonuclease activity in cell extracts of *E.coli* ER2426 carrying the *Pmel* endonuclease on the pRRS derived plasmid pRRSPmelR1 and the *Dra*I methylase 50 on the pACYC184 derived plasmid pACYC184DraIM10. 0.5 gram of cell paste was suspended in 1.5 ml of sonication buffer (20 mM Tris-HCl, 1 mM dithiothreitol, 0.1 mM EDTA, pH 7.5), broken by sonication and clarified by centrifugation. The extract was used to digest 1 µg of λDNA per 50 µl reaction volume in 1X NEBuffer 4, supplemented with 100 µg/ml BSA and incubation at 37°C for one hour. Lanes 1 and 10: *Hind*III-λ + *Hae*III-φX174 size standard; Lane 2: 0.01 µl crude extract; Lane 3: 5 x 10⁻³ µl crude extract; Lane 4: 2.5 x 10⁻³ µl crude extract; Lane 5: 1.25 x 10⁻³ µl crude extract; Lane 6: 6.25 x 10⁻⁴ µl crude extract; Lane 7: 3.13 x 10⁻⁴ µl crude extract; Lane 8: 1.56 x 10⁻⁴ µl crude extract; Lane 9: 55 7.81 x 10⁻⁵ µl crude extract. Total *Pmel* activity corresponds to approximately 1 x 10⁷ units per gram cell paste.

DETAILED DESCRIPTION OF THE INVENTION

[0018] The present invention relates to recombinant DNA which encodes the *Pmel* restriction endonuclease, as well as to methods of producing recombinant *Pmel* enzyme.

5 [0019] The cloning of the *Pmel* restriction endonuclease gene from *Pseudomonas mendocina* proved to be unusually difficult. The methylase selection method (U.S. Patent No. 5,200,333) was vigorously attempted but failed to yield a *Pmel* methylase producing clone. Even after cloning of the *Pmel* endonuclease gene, conserved methylase-motifs have not been located adjacent to the *Pmel* endonuclease gene. This lack of a *Pmel* methylase gene significantly complicated the cloning of the corresponding endonuclease gene.

10 [0020] In order to clone the *Pmel* restriction endonuclease gene, it was decided to obtain highly purified *Pmel* restriction endonuclease protein from *Pseudomonas mendocina* cells to determine amino acid sequence at the N-terminus of the endonuclease. The amino acid sequence was then used to design degenerate oligonucleotide primers for PCR amplification of this region of DNA from genomic *Pseudomonas mendocina* DNA. The sequence of the small PCR amplified DNA was then used to design non-degenerate inverse PCR primers, which were used to amplify DNA flanking the start of the *Pmel* endonuclease gene using inverse PCR technologies. Two such amplified products, a 1.4 kb product from *BssHII* digested and circularized *Pseudomonas mendocina* DNA template and a 900 bp product from *BsaWI* digested and circularized *Pseudomonas mendocina* DNA template were cloned into vector pUC19 and sequenced. The sequence data of an approximately 1.6 kb region of the *Pseudomonas mendocina* genome derived from the amplified products was used to identify the *Pmel* endonuclease gene. An open reading frame of 693 bp was observed to have N-terminal amino acid sequence matching that determined from sequencing the N-terminus of the purified *Pmel* endonuclease protein and was therefore identified to be the *Pmel* endonuclease gene. No obvious known methylase motifs were found in the six frame amino acid translations of both the 700 bp and 190 bp DNA sequence immediately 3' and 5' to the identified *Pmel* endonuclease gene respectively. In order to find a possible *Pmel* methylase, DNA sequence data beyond both sides of this 1.6 kb region needed to be obtained. To this end, additional inverse

15 PCR experiments were conducted. An additional 1.57 kb of DNA sequence 3' to the endonuclease and 3.1 kb of sequence 5' to the endonuclease were obtained and no obvious known methylase motifs were found. Several possibilities exist. One of the ORFs adjacent to the endonuclease gene may code for a *Pmel* methylase, but fail to contain highly homologous methylase motifs allowing it to be identified as such. The methylase could be quite large, and thus more sequence, particularly 3' to the endonuclease, might be needed to locate the gene. The methylase could be located elsewhere on the genomic *Pseudomonas mendocina* DNA and not adjacent to the endonuclease gene. Another possibility is that *Pseudomonas mendocina* DNA does not contain a methylase gene corresponding to the *Pmel* endonuclease.

20 [0021] Absent a *Pmel* methylase gene to express to protect the host from the corresponding endonuclease gene, other methylases that potentially could block *Pmel* restriction activity were investigated. *Msel* methylase, which recognizes TTAA, the internal four bases of the *Pmel* recognition sequence, was found to not block *Pmel* cleavage. A series of oligonucleotides containing a *Pmel* site was constructed so that one of the adenines or the cytosine in the recognition sequence was methylated and these oligos were subjected to *Pmel* cleavage to see if *Pmel* could be blocked by methylation. It was found that methylation at the third adenine residue blocked *Pmel* cleavage, but not methylation at either the first or second adenine, nor methylation at the C5 position of the cytosine residue. N4 cytosine methylation was not tested. The *DraI* methylase recognizes the DNA sequence 5'TTTAAA3' which is the internal six bases of the *Pmel* site 5'GTTTAAAC3'. The *DraI* methylase could methylate at any one of the three adenines and therefore might or might not block *Pmel* restriction digestion, so it was necessary to determine whether DNA modified by *DraI* methylase was resistant to *Pmel* restriction digestion. To this end, partial *Sau3AI* subclones of a *DraI* methylase producing clone, p*DraI*RM9.7-G2 were cloned into the vector pNEB193, which contains a single *Pmel* site and four *DraI* sites and *DraI* methylase producing clones were selected by the methylase selection method. The plasmids from these clones were isolated and shown to be protected from *Pmel* as well as *DraI* restriction. Since DNA modified by *DraI* methylase was protected from *Pmel* restriction digestion, the *Pmel* endonuclease gene could then be expressed in a host pre-protected by *DraI* methylase. *DraI* methylase was subsequently cloned into a vector, pACYC184, compatible with expression vectors such as pRRS or pAl17. This was accomplished by cloning *Sau3AI* partial subclones of p*DraI*RM9.7-G2 into pACYC184 and the selecting for methylase producing clones by methylase selection.

25 [0022] The *Pmel* endonuclease was then expressed by amplifying the complete gene from *Pseudomonas mendocina* DNA and cloning it into an expression vector, pRRS. This construct was introduced into a host which was pre-modified at *Pmel* sites by virtue of the *DraI* methylase gene carried on the separate compatible plasmid pACYC184. *Pmel* endonuclease is produced by growing the host containing the *Pmel* endonuclease gene and *DraI* methylase gene, inducing with the appropriate expression conditions, harvesting the cells and purifying the *Pmel* endonuclease.

30 [0023] The method described herein by which the *Pmel* restriction endonuclease is preferably cloned and expressed is illustrated in Figure 1 and includes the following steps:

35 [0024] 1. *Pseudomonas mendocina* is grown in flasks containing a modified LB medium with no magnesium added

at 37°C with agitation and aeration overnight. The cells are harvested, lysed and the genomic DNA purified.

[0025] 2. The *Pmel* restriction endonuclease is purified to near homogeneity from *Pseudomonas mendocina* cells by a combination of protein purification techniques developed at New England Biolabs, Inc. (see Example 1, step 2). The endonuclease so purified is nearly homogeneous on SDS polyacrylamide gel electrophoresis and has an apparent molecular weight of approximately 26 kilodaltons.

[0026] 3. The amino terminal amino acid sequence of the endonuclease is obtained using an Applied BioSystems Division, Perkin-Elmer Corporation (Foster City, California) 470A Protein Sequencer (Waite-Rees, et al., *J. Bacteriol.* 173:5207-5219 (1991)), and used to direct synthesis of degenerate oligonucleotide primers for amplification of the DNA at the start of the *Pmel* endonuclease gene from *Pseudomonas mendocina* genomic DNA, and to identify the *Pmel* endonuclease gene in subsequent studies.

[0027] 4. A portion of the *Pmel* endonuclease gene is amplified using degenerate DNA primers based on the amino acid sequence obtained in step 3, one corresponding to the amino acid residues 1 through 6 (M)TTNSP (SEQ ID NO: 1) and one corresponding to amino acid residues 10 through 14 MIDEc (SEQ ID NO:2) for the reverse strand of DNA. Individual clones of the amplified DNA are sequenced.

[0028] 5. *Pseudomonas mendocina* genomic DNA is digested by *Apol*, *BsaHI*, *BsrFI*, *BstYI*, *Eael*, *HaeII*, *Sau3AI*, *NlaIII*, *EagI* and *BssHII* endonucleases and the resulting fragments are ligated at low DNA concentration to favor intramolecular ligation. Primer Pmel-IP1 is designed to anneal in the region between the degenerate primers of step 4 corresponding to amino acid residues Asp-Val-Gly-Met-Ile-Asp (SEQ ID NO:3) and the first nucleotide G of the codon coding for Glu with its 3' end oriented toward the unknown region. Since the DNA sequences of clones obtained in

step 4 differed in sequence at the fourth and fifth residues, Asn and Ser, two separate primers are made with primer Pmel-IP2 hybridizing to one class of sequence and primer Pmel-IP3 to the second class of sequence. The circularized fragments containing DNA corresponding to the N-terminus of the *Pmel* endonuclease gene are amplified using primers Pmel-IP1 and Pmel-IP2 and primers Pmel-IP1 and Pmel-IP3. Amplified products of various sizes are produced and cloned into pUC19 vector.

[0029] 6. The various pUC19 constructs are sequenced. Only one construct, pUC19BssHII21 containing a 1400 bp amplified fragment from *BssHII* digested and circularized DNA contains DNA sequence that matches the N-terminal amino acid sequence of the *Pmel* endonuclease 3' to primer Pmel-IP1. However, primer Pmel-IP3 hybridizes elsewhere likely due to mismatches on the primer sequences. It follows that both primers Pmel-IP2 and Pmel-IP3 are likely to contain mismatches at the sequence for the fourth and fifth residues Asn and Ser.

[0030] 7. A new set of primers are designed based on the sequence obtained from the pUCBssHII21 clone. The circularized fragments containing DNA corresponding to the N-terminus of the *Pmel* endonuclease gene are amplified using the new primers. The *BsaWI* digested and circularized DNA produces a 900 bp product which is cloned into the vector pUC19.

[0031] 8. The construct, pUC19BsaW15 containing the amplified 900 bp fragment from the *BsaWI* digested circular DNA is sequenced. There is an open reading frame matching the N-terminal amino acid sequence data and which is of the correct size to produce a protein of approximately 26 kD. The *BsaWI* product contains 190 bp 5' to the endonuclease gene and 90 bp 3' to the endonuclease gene. The *BssHII* product contains 700 bp 3' to the endonuclease gene. No obvious methylase motifs are found in the sequenced region flanking the endonuclease gene. To search for a *Pmel* methylase gene, DNA flanking both sides of the endonuclease gene is amplified, cloned and sequenced as described in the following sections.

[0032] 9. *Pseudomonas mendocina* DNA 5' to the *Pmel* endonuclease gene is amplified in inverse PCR reactions using primers that hybridize near the 5' end of the endonuclease gene. The restriction endonuclease digested and circularized *Pseudomonas mendocina* DNA prepared in section 5 is used as templates. Amplified products are cloned into pUC19 vector.

[0033] 10. Various pUC19 constructs carrying the amplified products from section 9 are sequenced. *BstYI* digested and circularized DNA produces a 3.3 kb product and *HaeII* digested and circularized DNA produces a 0.9 kb product. No obvious methylase motifs can be found in the six frame amino acid translations from approximately 3.1 kb of sequenced DNA 5' to the *Pmel* endonuclease gene.

[0034] 11. *Pseudomonas mendocina* DNA 3' to the *Pmel* endonuclease gene is amplified in two consecutive inverse PCR experiments. *Pseudomonas mendocina* DNA is digested with *AatII*, *HincII*, *MscI*, *MseI*, *SacI*, *StuI* and *Tsp509I* endonucleases and the resulting fragments are ligated at low DNA concentration to favor intramolecular ligation. These circular templates as well as the *BsaHI* digested, circular template from section 5 are used in the first inverse PCR experiment. One of the primers hybridizes to DNAs just outside the 3' terminus of the *Pmel* endonuclease gene with its 3' end oriented toward the unknown region. The other hybridizes within and near the 3' end of endonuclease gene and is oriented in the opposite direction. The *AatII* digested, circularized DNA produces a 1.1 kb product; the *MscI* digested, circular DNA produces a 1.4 kb product, and the *BsaHI* digested, circular DNA produces a 0.7 kb product. These amplified DNAs are cloned into pUC19 and sequenced. Two more primers based on the newly derived DNA sequence information are synthesized and used in the second inverse PCR experiment. The *HaeII* digested, circularized

DNA (section 5) produces a 0.7 kb product which is cloned into pUC19 and sequenced. Approximately 1.57 kb of DNA 3' to the 3' end of the endonuclease gene thus sequenced contains no obvious methylase motifs.

[0035] 12. A *Dra*I methylase producing clone, pDraIRM9.7-G2 is partially digested with *Sau*3AI and the resulting DNA fragments are inserted into pNEB193 vector. Transformants are pooled together, the populations of plasmids are purified and digested with *Dra*I endonuclease. The restricted plasmids are transformed back into *E.coli* to recover uncut clones. Plasmids of nine individual transformants surviving *Dra*I digestion are purified. Eight of the nine plasmids are protected from *Dra*I endonuclease digestion as well as from *Pme*I endonuclease digestions.

[0036] 13. Overexpressing the *Pme*I endonuclease gene:

10 A. General considerations:

[0037] There are a number of ways in which the restriction gene can be overexpressed. The DNA sequence and detailed mapping information help determine the best approach for overexpression of the restriction endonuclease gene.

15 [0038] One approach for overexpression comprises designing primers that hybridize directly at the N-terminus of the restriction endonuclease gene and somewhere downstream (3') of the gene in order to use the polymerase-chain reaction to amplify the entire endonuclease gene. The resulting DNA fragment can be inserted into an expression vector such as pRRS directly downstream of an inducible promoter (lacUV5).

20 [0039] Alternatively, overexpression can be accomplished by inserting a promoter recognized strongly by *E. coli*, such as *P_{lac}* on pAGR3 (New England Biolabs, Inc.; Beverly, Massachusetts) directly in front of the beginning of the restriction endonuclease gene. This may be accomplished by finding convenient restriction sites near the beginning and end of the restriction endonuclease gene and compatible restriction sites near the promoter of pAGR3, and transferring the restriction gene into pAGR3 in line with the *P_{lac}* promoter. Other regulated promoters which can be used are *P_{lac}UV5* (Fuller, *Gene* 19:43-54 (1982)), and *IPL* (Shimatake and Rosenberg, *Nature* 254:128 (1981)) on pUC19 and pBR322 derivatives. In addition, a strong ribosome binding site (Shine & Dalgarno, *Proc. Natl. Acad. Sci. USA* 71: 342-1346 (1974)) can be placed in front of the gene to increase expression.

25 [0040] To obtain a stable clone which overexpresses the restriction endonuclease, the host is generally pre-protected from restriction endonuclease digestion. In the present invention this is accomplished by cloning the *Dra*I methylase on a separate plasmid. DNA modified by *Dra*I methylase is shown in this application to be resistant to *Pme*I endonuclease digestion. The plasmid used must be compatible with the expression vector. The methylase also must be produced at a level which will protect the host's genome from digestion by the overexpressed restriction endonuclease gene.

30 [0041] The DNA sequence of the gene can be altered by site-directed mutagenesis or by resynthesizing the gene itself to use codons that are more efficiently utilized in *E. coli* (Ikemura, *J. Mol. Biol.* 151:389-409 (1981)).

35 B. Cloning the *Dra*I methylase in a compatible vector:

[0042] The *Sau*3AI partially digested pDraIRM9.7-G2 DNA fragments from section 12 are ligated into vector pACYC184 previously cleaved with *Bam*HI and dephosphorylated. Transformants are pooled together, the populations of plasmids are purified and digested with *Dra*I endonuclease. The restricted plasmids are transformed back into *E. coli* to recover uncut clones. Plasmids of twelve of the fourteen individual transformants mini-prepped are protected from *Dra*I endonuclease digestion. One such clone designated as pACYC184DraIM10 containing an insert of approximately 3 kb is used for subsequent *Pme*I endonuclease expression.

40 C. Expression of *Pme*I endonuclease:

[0043] DNA primers are designed and synthesized to amplify the *Pme*I endonuclease gene. The forward primer has the following elements: a *Pst*I site to facilitate cloning, a stop codon in frame with the *lacZ* gene, an *E.coli* consensus strong ribosome binding site, 7 nucleotide spacer sequence between the ribosome binding site and the ATG start codon of the *Pme*I endonuclease and 24 nucleotides matching the *Pme*I endonuclease DNA sequence for hybridization. The reverse primer has a *Bgl*II site and 21 nucleotides matching *Pseudomonas mendocina* DNA 84 bp 3' to the 3' end of *Pme*I endonuclease gene for hybridization. The endonuclease gene is amplified from the genomic DNA using these primers. The amplified DNA is cleaved by *Bgl*II and *Pst*I and ligated into the expression vector pRRS, which has been previously cleaved by *Pst*I and *Bam*HI endonucleases and gel purified. The ligation reaction is transformed into *E.coli* ER2426 competent cells containing pACYC184DraIM10. Vectors containing inserts of desired size are identified by miniprep procedures. Several clones are grown to mid-log phase and induced with 0.5 mM IPTG for 16 hours. The cells are then harvested by centrifugation, resuspended in sonication buffer, lysed by sonication and the extract assayed for *Pme*I endonuclease activity. One such *Pme*I expressing host, designated pRRSPmeIR1/pACYC184DraIM10, is

propagated and used to produce *Pmel* restriction endonuclease.

[0044] 14. Production: The *Pmel* endonuclease may be produced from host cells carrying the overexpressed *Pmel* restriction endonuclease gene by propagation in a fermenter in a rich medium with the appropriate antibiotic selection and induction. The cells are thereafter harvested by centrifugation and disrupted by sonication to produce a crude cell extract containing *Pmel* restriction endonuclease activity.

[0045] 15. Purification: The crude cell extract containing the *Pmel* endonuclease is purified by a combination of protein purification techniques, such as affinity-chromatography or ion-exchange chromatography.

[0046] Although the above-outlined steps represent the preferred mode for practicing the present invention, it will be apparent to those skilled in the art that the above described approach can vary in accordance with techniques known in the art.

[0047] The following Example is given to illustrate embodiments of the present invention as it is presently preferred to practice. It will be understood that this Example is illustrative, and that the invention is not to be considered as restricted thereto except as indicated in the appended claims.

[0048] The references cited above and below are herein incorporated by reference.

15

EXAMPLE 1

CLONING OF THE *PME* RESTRICTION ENDONUCLEASE GENES

20 [0049] 1. DNA purification: 5 g of *Pseudomonas mendocina* (NEB #698) cell paste was resuspended by gentle shaking in 20 ml of 25% sucrose, 0.05 M Tris-HCl, 1 mM EDTA, pH 8.0. 5 ml of 0.5 M EDTA, pH 8.0 and 6 ml of freshly prepared 10 mg/ml lysozyme in 0.25 M Tris-HCl pH 8.0 was added and the solution was incubated at 4°C for 2 hours. 24 ml of Lysis mix (1% Triton-X100, 50 mM Tris, 62.5 mM EDTA, pH 8.0) was added followed by 5 ml of 10% SDS and the solution was incubated at 4°C overnight. The solution was extracted with 50 ml of equilibrated phenol, the aqueous phase was recovered and extracted with 50 ml of chloroform two times. The aqueous solution was dialyzed against four changes of 2 L of 10 mM Tris, 1 mM EDTA, pH 8.0 overnight. The dialyzed solution was then digested with RNase (100 ug/ml) at 37°C for 1 hour. The DNA was precipitated by the addition of 1/10th volume 5M NaCl and 0.55 volume of 2-propanol and spooled onto a glass rod. The DNA was air dried and then dissolved in 10 ml of 10 mM Tris, 1 mM EDTA, pH 8.0.

25 [0050] 2. Purification of the *Pmel* restriction endonuclease from *Pseudomonas mendocina* (NEB #698): *Pmel* restriction enzyme may be produced from NEB #698 by propagation of *Pseudomonas mendocina* cells in a modified LB medium containing no magnesium at 37°C with agitation and aeration overnight. The cells were harvested by centrifugation. All of the following procedures were performed on ice or at 4°C. 196 g of cell pellet (wet weight) was resuspended in 600 ml of buffer A (20 mM Tris-HCl, 1 mM Dithiothreitol (DTT), 0.1 mM EDTA, 5% glycerol, pH 7.4) containing

35 50 mM NaCl (buffer A.05), followed by an addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 25 ug/ml. The cell suspension was then broken by sonication. The extract was centrifuged at 12 K rpm for 90 minutes and the supernatant was collected. The pellets were rinsed with 50 ml of buffer A.05 and centrifuged again at 15 K rpm for 90 minutes. The supernatant was combined together (700 ml total) and loaded onto a 216 ml Q-sepharose column (Pharmacia; Piscataway, NJ) equilibrated with buffer A.05. This column was washed with 200 ml of buffer A.

40 05 and the *Pmel* activity was eluted with the flow through and the wash. *Pmel* containing fractions were pooled together and loaded onto a 216 ml heparin-sépharose column (Pharmacia; Piscataway, NJ) equilibrated in buffer A.05, washed with 600 ml of buffer A.05 and then a 2 L linear gradient from 50 mM to 800 mM NaCl in buffer A was formed. 21 ml fractions were collected. Fractions were assayed for *Pmel* restriction activity with lambda DNA and the midpoint of eluted activity was found to be at approximately 480 mM NaCl concentration. A total of 12 fractions were pooled and the amount of enzyme activity was estimated to be 750,000 units. This heparin-sepharose pool was applied to a 12 ml hydroxylapatite (HPT) column (Calbiochem; LaJolla, CA) equilibrated in buffer B (20 mM KPO₄, 6 mM 2-mercaptoethanol, 0.1 mM EDTA, 5% glycerol, pH 6.8) containing 50 mM NaCl (buffer B.05), washed with 36 ml of buffer B.05 and followed by a 120 ml linear gradient from 50 mM NaCl to 1 M NaCl in buffer B. 1.2 ml fractions were collected. Fractions were assayed for *Pmel* activity with lambda DNA. The midpoint of restriction enzyme activity eluted at approximately 500 mM NaCl. A total of 13 fractions were pooled and dialyzed against buffer C (20 mM KPO₄, 1 mM DTT, 0.1 mM EDTA, 5% glycerol, pH 6.8) contain 50 mM NaCl (buffer C.05) overnight. The dialyzed solution was applied to a 1 ml polyCat A column (Custom LC, Inc.; Houston, TX) equilibrated in buffer C.05, followed by a 50 ml linear gradient from 50 mM NaCl to 500 mM NaCl in buffer C. 1 ml fractions were collected and the fractions were assayed for restriction enzyme activity with lambda DNA. The *Pmel* activity eluted at approximately 270 mM NaCl. 3 fractions

45 were pooled together, diluted with 5 volumes of buffer A and loaded onto a 1 ml Mono Q FPLC column (Pharmacia; Piscataway, NJ) equilibrated with buffer A.05. The column was washed with 2 ml of buffer A.05 and the *Pmel* activity eluted with the flow through and the wash. *Pmel* containing fractions were pooled together and loaded directly onto a 3 ml Heparin-TSK FPLC column (TosoHaas; Philadelphia, PA) equilibrated with buffer A.05. The column was washed

with 2 ml of buffer A.05 followed by a 60 ml linear gradient of 50 mM NaCl to 500 mM NaCl in buffer A. 1 ml fractions were collected and the *Pmel* activity was assayed with lambda DNA. The peak of restriction enzyme activity eluted at 300 mM NaCl. The amount of *Pmel* restriction enzyme activity purified was estimated to be 116,000 units. The peak fraction was loaded onto an SDS-PAGE protein gel and subjected to electrophoresis. The gel was stained with Coomassie blue R-250 and a prominent band at approximately 26 kD corresponding to the *Pmel* restriction endonuclease activity was observed.

[0051] 3.. Amino-Terminal *Pmel* protein sequence: The *Pmel* restriction-endonuclease, prepared as described in section 2 above, was subjected to electrophoresis and electroblotted according to the procedure of Matsudaira (Matsudaira, P., J. Biol. Chem. 262:10035-10038 (1987)), with modifications as previously described (Looney, et al., Gene 80:193-208 (1989)). The membrane was stained with Coomassie blue R-250 and the protein band of approximately 26 kD was excised and subjected to sequential degradation on an Applied BioSystems Division, Perkin-Elmer Corporation (Foster City, California) Model 407A gas phase protein sequencer (Waite-Rees, et al., J. Bacteriol. 173: 5207-5219 (1991)). The first 24 residues of the 27 kD protein corresponded to (Met)-Thr-Thr-Asn-Ser-Pro-Ser-Asp-Val-Gly-Met-Ile-Asp-Glu-Cys-Leu-Ser-Ile-Val-Xaa-Thr-Xaa-Leu-Ala (SEQ ID NO:4).

[0052] 4. Amplification of N-terminal *Pmel* DNA: A portion of the *Pmel* endonuclease gene is amplified using degenerate DNA primers based on the amino acid sequence obtained in step 3. Two primers corresponding to the amino acid residues 1 through 6 (M)TTNSP (SEQ ID NO:1) are synthesized: *Pmel*-PS1 5' GTTGGATCCATGACNACNAAYTC-NCC 3' (SEQ ID NO:5) and *Pmel*-PS2 5' GTTGGATCCATGACNACNAAYAGYCC 3' (SEQ ID NO:6). A primer corresponding to amino acid residues 10 through 14 MIDEK, *Pmel*-PS3 5' GTTCTGCAGRCAYTCRTCDATCAT 3' (SEQ ID NO:7) is synthesized to hybridize at the reverse strand of DNA. A reaction mix was made by combining:

30 ul of 10X Vent® reaction buffer
 18 ul of 4 mM dNTP solution
 15 ul of primer *Pmel*-PS1
 25 15 ul of primer *Pmel*-PS2
 3 ul of *Pseudomonas mendocina* DNA (approximately 600 ng)
 219 ul dH₂O
 6 ul (12 units) of Vent® Exo- polymerase (NEB#257)

[0053] The mix was split into three 94 ul aliquots; to the first is added 6 ul of dH₂O, to the second is added 3 ul of dH₂O and 3 ul of 100 mM MgSO₄ (5 mM Mg⁺⁺ final concentration) and to the last is added 6 ul 100 mM MgSO₄ (8 mM Mg⁺⁺ final concentration). The PCR amplification conditions are: 95°C for 2 minutes for one cycle, followed by 20 cycles of 95°C for 30 seconds, 40°C for 30 seconds and 72°C for 5 seconds. The PCR amplified DNA is size selected on a 3.5% NuSieve agarose gel and cloned into pUC19. Individual clones of the amplified DNA are sequenced. Clones containing both *Pmel*-PS1 and *Pmel*-PS2 are observed, leaving the codon usage for amino acid five, serine, in question.

[0054] 5. Cloning DNA adjacent to the N-terminal end of *Pmel* restriction endonuclease gene: Template preparation for inverse PCR amplification: 3 ug of *Pseudomonas mendocina* DNA was digested with 20 units of *ApoI* restriction endonuclease in 100 ul 1X NEBuffer #3 (50 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl, 1 mM DTT, pH 7.9) for 1 hour at 50°C. The restriction digestion mixture was extracted with one volume of equilibrated phenol:chloroform (50:50, v/v) and the aqueous phase was recovered and extracted with one volume of chloroform two times. The DNA was precipitated by the addition of 1/10th volume of 5 M NaCl and 1 volume of 2-propanol and washed with 70% ice cold ethanol. The DNA was resuspended in 50 ul of 1X TE. 10 ul of this digested *Pseudomonas mendocina* DNA (approximately 0.5 ug) was circularized in 500 ul 1X ligase buffer using 2000 U of T4 DNA ligase at 16°C overnight. A portion of this circularization reaction was used as DNA template for subsequent inverse PCR reactions. Circularized *Bsa*H*I*, *Bsr*F*I*, *Bst*Y*I*, *Eae**I*, *Haell*, *Sau*3*A**I*, *Nla*III, *Eag**I* and *Bss*H*II* digested *Pseudomonas mendocina* DNA were prepared in the same manner.

[0055] One forward or coding strand primer, *Pmel*-IP1 and two reverse or noncoding strand primers, *Pmel*-IP2 and *Pmel*-IP3 of sequences shown below were synthesized based on DNA sequences obtained from step 4. Primer *Pmel*-IP1 was designed to hybridize nucleotides corresponding to amino acid residues 8 to 13 Asp-Val-Gly-Met-Ile-Asp (SEQ ID NO:8) and the first nucleotide G of the codon coding for residue 14 Glu. A *Bam*H*I* restriction site was also included in this primer to facilitate cloning. Primer *Pmel*-IP2 was designed to hybridize the last two nucleotides of the codon coding for residue 2 Thr, nucleotides corresponding to amino acid residues 3 to 7 Thr-Asn-Ser-Pro-Ser (SEQ ID NO:9) and the first nucleotide of the codon coding for residue 8 Asp. A *Pst**I* restriction site was also included in this primer to facilitate cloning.

Primer Pmel-IP1

5 5'-GTTGGATCCGACGTCGGCATGATCGACG-3' (SEQ ID NO:10)

10 Primer Pmel-IP2

5'-GTTCTGCAGCTGAGGGCTGTCGTTG-3' (SEQ ID NO:11)

15 Primer Pmel-IP3

5'-GTTCTGCAGCTGAGGGCGAATTCTGTTG-3' (SEQ ID NO:12)

20 [0056] It is often convenient to directly insert PCR fragments into vectors previously cleaved by restriction enzymes that produce blunt ends such as *Sma*I. This requires that the PCR fragments to be inserted to have phosphate groups at the 5' ends. To this end, 15 μ l of the DNA primer Pmel-IP1 (200 μ M) was phosphorylated by 20 units of T4 polynucleotide kinase in 1X T4 polynucleotide kinase buffer (70 mM Tris-HCl, 10 mM MgCl₂, 5 mM dithiothreitol, pH 7.6) supplemented with 1 mM ATP in a 100 μ l reaction volume at 37°C for one hour. Primers Pmel-IP2 and Pmel-IP3 were also phosphorylated at the 5' end as described above. The T4 DNA polynucleotide kinase was heat inactivated by incubating at 65°C for 20 minutes. The phosphorylated primers were diluted with 2 volumes of 1X TE to a final concentration of 10 μ M and were used in the following PCR reactions.

25 [0057] Inverse PCR reactions were performed with digested, circularized *Pseudomonas mendocina* DNA as templates using the phosphorylated primers Pmel-IP1 and Pmel-IP2. Three different Mg⁺⁺ concentration was used in inverse PCR reactions against an individual DNA template. A reaction mix was made by combining:

30 30μ l of 10X Vent® reaction buffer
 18 μ l of 4 mM dNTP solution
 35 15 μ l of phosphorylated primer Pmel-IP1
 15 μ l of phosphorylated primer Pmel-IP2
 36 μ l of circularized DNA template (approximately 25 ng)
 168 μ l dH₂O
 6 μ l (12 units) of Vent® Exo- polymerase NEB#257

40 [0058] The mix was split into three 94- μ l aliquots; to the first was added 6 μ l of dH₂O, to the second was added 3 μ l of dH₂O and 3 μ l of 100 mM MgSO₄ (5 mM Mg⁺⁺ final concentration) and to the last was added 6 μ l 100 mM MgSO₄ (8 mM Mg⁺⁺ final concentration). The PCR amplification conditions were: 95°C for 2 minutes for one cycle, followed by 5 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 2.5 minutes, followed by 20 cycles of 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 2.5 minutes. 15 μ l of the PCR reaction was analyzed by electrophoresis on a 0.8% agarose gel. Among some unspecific amplified DNA background, the following products were observed: a 1700 bp fragment and two closely spaced fragments around 1500 bp from *Apo*/digested circular template, a 1500 bp fragment from *BsYI* digested circular template, a 900 bp fragment and a 750 bp fragment from *Bsa*HII digested circular template and a 2500 bp fragment from *Eag*/digested circular template. These amplified product were gel purified. Gel purification: 100 μ l of PCR reactions were electrophoresed into 1% LMP agarose, bands were excised from the gel, melted at 65°C for five minutes, cooled to 40°C for 5 minutes and the agarose was digested by the addition of 5 units of β -agarase (New England Biolabs #392) with incubation at 40°C for 1 hour. It was then extracted with one volume of equilibrated phenol:chloroform (50:50, v/v) and the aqueous phase was recovered and extracted with one volume of chloroform two times. The DNA was precipitated by the addition of 1/10 volume of 5 M NaCl and 2 volumes of 2-propanol, washed with 70% ethanol, air dried and resuspended in 50 μ l of 1XTE buffer.

55 [0059] Inverse PCR reactions were also performed with phosphorylated primers Pmel-IP1 and Pmel-IP3 using the same set of templates in the similar manner as described above. 15 μ l of the PCR reaction was analyzed by electrophoresis on a 0.8% agarose gel. The following fragments were gel purified: a 2000 bp, a 1800 bp and a 870 bp fragments

from *Apol* digested circular template, a 1350 bp fragment from *Bs/YI* digested circular template, a 1200 bp fragment *BsrFI* digested circular template, a 2100 bp and a 1400 bp fragments from *BssHII* digested circular template, a 2500 bp fragment from *EagI* digested circular template, and a 800 bp fragment from *NlaIII* digested circular template.

5 [0060] Each of the gel purified amplified DNA product was ligated into the vector pUC19 respectively as follows: 5 *ul* of gel purified DNA was ligated to 100 ng of pUC19 vector previously cleaved with *SmaI* and dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIP) in a final volume of 20 *ul* in 1X T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 ug/ml BSA, pH 7.5) with 400 U T4 DNA ligase. 10 *ul* of the ligation mixture was then transformed into *E. coli* strain ER2267 and plated on L-broth plates containing 100 ug/ml ampicillin, 40 ug/ml Xgal and 50 ug/ml IPTG for individual colonies. White colonies were individually picked to inoculate 10 ml of L-broth containing 100 ug/ml ampicillin and were allowed to propagate at 37°C overnight. Clones of the desired construct were identified by performing minipreps, digesting the purified DNA and analyzing it by agarose gel electrophoresis.

10 [0061] Miniprep Procedure: Each culture was centrifuged at 8000 rpm for 5 minutes; the supernatant was discarded and the cell pellet was resuspended in 1.0 ml of 25 mM Tris, 10 mM EDTA, 50 mM glucose, pH 8.0. After 10 minutes at room temperature, 2 ml of 0.2 M NaOH, 1% SDS was added to each tube and the tubes were shaken to lyse the 15 cells and then placed on ice. Once the solutions had cleared, 1.5 ml of 3 M sodium acetate pH 4.8, was added to each and shaken. The precipitates that formed were spun down at 15,000 rpm, 4°C for 10 minutes. Each supernatant was poured and mixed. After 10 minutes at room temperature, the tubes were spun at 15,000 rpm for 10 minutes to pellet the precipitated nucleic acids. The supernatants were discarded and the pellets were air-dried at room temperature for 30 minutes. Once dry, the pellets were dissolved in 850 *ul* of 10 mM Tris pH 8.0, 1 mM EDTA, containing 100 ug/ml RNase and incubated for 1 hour at 37°C to digest the RNA. The DNA was precipitated by the addition of 85 *ul* of 5 M NaCl followed by 600 *ul* of isopropanol. After 10 minutes at room temperature the DNA was spun down by centrifugation for 5 minutes, the supernatants were discarded, the pellets were dried and then redissolved in a final solution of 150 *ul* of 10 mM Tris, 1 mM EDTA pH 8.0 (1X TE). The plasmid minipreps were subsequently analyzed by digestion with various restriction endonucleases.

20 [0062] 6. DNA Sequencing: DNA sequencing was performed using an ABI 373 automated sequencing system according to the manufacturer's instructions, using M13/pUC primers #1233 and #1224 (NEB). Miniprep DNA preparations of various pUC19 constructs containing DNA fragments from inverse PCR reactions described in section 5 were used as templates. Only one clone had sequence matching the *Pmel* endonuclease amino acid sequence; a 1400 bp fragment amplified from *BssHII* digested circular template in a inverse PCR reaction using primers Pmel-IP1 and Pmel-IP3. This clone was designated as pUC19BssHII21. The DNA sequence at one end of this clone matched with the portion of the *Pmel* endonuclease sequence 3' from the primer Pmel-IP1. However, the DNA sequence from the other end did not match the portion of the *Pmel* endonuclease sequence 3' from the primer Pmel-IP3. It was concluded that primer Pmel-IP1 hybridized at the desired position on *Pmel* genomic DNA and primer Pmel-IP3 hybridized elsewhere on the *Pmel* genomic DNA, likely due to mismatches on the primer sequence. Since there were discrepancies in the 35 codon sequences for fourth and fifth residues, Asn and Ser, a new set of inverse PCR primers was designed to exclude DNA sequences coding for these residues.

30 [0063] 7. Inverse PCR amplifications using new set of primers to amplify and clone DNA adjacent to N-terminus of the endonuclease gene: Primers Pmel-IP4 and Pmel-IP5 were designed according to the DNA sequences obtained in step 6. Primer Pmel-IP4 included nucleotides corresponding to amino acid residues Val-Gly-Met-Ile-Asp (SEQ ID NO:13) and the first two nucleotides of the codon for Glu. A *BamHI* site was also included in this primer to facilitate 40 cloning.

45 Primer Pmel-IP4

5'-GTTGGATCCGTCGGCATGATCGACGA-3' (SEQ ID NO:14)

50 [0064] Primer Pmel-IP5 was designed to hybridize with the first two nucleotides of codon for Gly and nucleotides corresponding to amino acid residues Val-Asp-Ser-Pro. A *BamHI* site was also included in this primer to facilitate cloning.

55 Primer Pmel-IP5

5'-CATGGATCCGACGTCTGAGGG-3' (SEQ ID NO:15)

[0065] Inverse PCR reactions were carried out using *Bsa*WI digested, circularized *Pmel* DNA as the template. In the reaction that was successful in amplifying the product, a reaction mix was made by combining:

- 5 10 ul of 10X Vent® reaction buffer
- 6 6 ul of 4 mM dNTP solution
- 3 ul of 100 mM MgSO₄ (5 mM Mg⁺⁺ final concentration)
- 5 ul of phosphorylated primer Pmel-IP4
- 5 ul of phosphorylated primer Pmel-IP5
- 12 ul of circularized DNA template (approximately 25 ng)
- 10 58 ul dH₂O
- 2 ul (4 units) of Vent® Exo⁻ polymerase NEB#257

[0066] The PCR amplification conditions were: 95°C for 2 minutes for one cycle, followed by 5 cycles of 95°C for 30 seconds, 40°C for 1 minute and 72°C for 2.5 minutes, followed by 20 cycles of 95°C for 30 seconds, 60°C for 1 minute and 72°C for 2.5 minutes. 15 ul of the PCR reaction was analyzed by electrophoresis on a 0.8% agarose gel.

[0067] A 900 bp product was observed, gel purified and resuspended in 50 ul of 1X TE. 5 ul of this DNA was digested with 20 U of *Bam*HI (NEB#136) in a final volume of 20 ul in 1X *Bam*HI Buffer (150 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol, pH 7.9)) at 37°C for 1 hour. The restriction endonuclease *Bam*HI was heat inactivated by incubating the reaction mixture at 78°C for 20 minutes. 5 ul of the digested DNA was ligated to 100 ng of the vector pUC19 previously cleaved with *Bam*HI, dephosphorylated with CIP and agarose gel purified, using 400 U of T4 DNA ligase in 20 ul final volume of 1X T4 DNA ligase buffer at 16 °C for 2 hours. 10 ul of the ligation mixture was transformed into *E.coli* strain ER2267 and plated on L-broth plates containing 100 ug/ml ampicillin, 40 ug/ml Xgal and 50 ug/ml IPTG for individual colonies. White colonies were individually picked to inoculate 10 ml of L-broth containing 100 ug/ml ampicillin and were allowed to propagate at 37°C overnight. Clones of the desired construct were identified by performing minipreps, digesting the purified DNA and analyzing it by agarose gel electrophoresis. One clone, designated as pUC19BsaW15, containing the 900 bp fragment was sequenced.

[0068] 8. DNA sequencing of the clone pUC19BsaW15: DNA sequencing was performed using an ABI 373 automated sequencing system according to the manufacturer's instructions, using M13/pUC primers #1233 and #1224 (New England Biolabs, Beverly, MA) and miniprep DNA preparation of pUC19BsaW15 as template. The DNA sequence at one end of this clone matched with the portion of the *Pmel* endonuclease sequence corresponding to and 3' from the primer Pmel-IP4. The DNA sequence from the other end also matched the portion of the *Pmel* endonuclease sequence corresponding to and 3' from the primer Pmel-IP5. The codon sequences for residues 4 (Asn) and 5 (Ser) were found to be 5' AAC 3' and 5' TCC 3'. An open reading frame of 693 bp with its N-terminal amino acid sequence matching the N-terminal protein sequence data obtained in section 3 was identified as the *Pmel* restriction endonuclease gene. The six frame amino acid sequence translated from the 700 bp DNA sequence immediately 3' to the endonuclease gene were compared to the conserved amino acid motifs of various types of known methylases and no amino acid sequences homologous to these motifs could be identified. Similarly, no obvious methylase motifs could be found from the six frame amino acid sequence translated from the 200 bp DNA sequence immediately 5' adjacent to the endonuclease gene. In order to locate a possible *Pmel* methylase gene, DNA region located further away from both sides of the endonuclease gene was amplified, cloned and sequenced in the subsequent steps.

[0069] 9. Cloning DNA 5' to the *Pmel* endonuclease gene: The following restriction endonuclease digested, circularized *Pseudomonas mendocina* DNA from section 5 were used as templates for inverse PCR amplification: *Apa*I, *Eag*I, *Bss*HII, *Bsr*F, *Hae*II and *Bst*YI. Primers Pmel-IP6 and Pmel-IP7 shown below were designed to anneal within and toward the 5' end of the *Pmel* endonuclease gene. Both primers contain a *Pst*I site to facilitate cloning.

45

Primer Pmel-IP6

50 5'-phospho-GTTCTGCAGCTCGTTGTCTTCTTCTGC-3'

(SEQ ID NO:16)

55

Primer Pmel-IP7

5'-phospho-ATCCTGCAGGCGACGTTGGACGATG-3'
(SEQ ID NO:17)

10 [0070] In the reaction that was successful in amplifying the product, a reaction mix was made by combining: 10 ul of 10X Vent® reaction buffer (containing 2 mM Mg⁺⁺ final concentration)

15 6 ul of 4 mM dNTP solution
 5 ul of phosphorylated primer Pmel-IP6
15 5 ul of phosphorylated primer Pmel-IP7
 12 ul of circularized DNA template (approximately 25 ng)
 58 ul dH₂O
 2 ul (4 units) of Vent® Exo- polymerase NEB#257

20 [0071] The PCR amplification conditions were: 95°C for 2 minutes for one cycle, followed by 5 cycles of 95°C for 20 seconds, 50°C for 1 minute and 72°C for 2.5 minutes, followed by 20 cycles of 95°C for 30 seconds, 65°C for 1 minute and 72°C for 2.5 minutes. 15 ul of the PCR reaction was analyzed by electrophoresis on a 0.8 % agarose gel.

[0072] Among some nonspecific amplified background DNA, a 1.8 kb product was observed in the *Apol* circular template PCR reaction; a 1.6 kb product was observed in the *BssHII* circular template PCR reaction; a 3.3 kb product was observed in the *BstYI* circular template PCR reaction; a 1.6 kb product was observed in the *EagI* circular template PCR reaction, and a 0.9 kb product was observed in the *HaeIII* circular template PCR reaction. These five products were gel purified and resuspended in 60 ul of 1XTE (*BstYI* product was resuspended in 20 ul of 1XTE). 5 ul of gel purified *Apol* product was ligated to 100 ng of the vector pUC19 previously cleaved with *SmaI*, dephosphorylated with CIP and agarose gel purified, using 400 U of T4 DNA ligase in 20 ul final volume of 1X T4 DNA ligase buffer at 16°C for 2 hours. The gel purified *BssHII*, *EagI*, *HaeIII* and *BstYI* products were similarly ligated into pUC19 as described above. 10 ul of each ligation mixture was transformed into *E.coli* strain ER2267 and plated on L-broth plates containing 100 ug/ml ampicillin, 40 ug/ml Xgal and 50 ug/ml IPTG for individual colonies. White colonies were individually picked to inoculate 10 ml of L-broth containing 100 ug/ml ampicillin and were allowed to propagate at 37°C overnight. Clones of the desired construct were identified by performing minipreps, digesting the purified DNA and analyzing it by agarose gel electrophoresis.

[0073] 10. Sequencing DNA 5' to the endonuclease gene: The constructs with desired inserts from section 9 were sequenced with M13/pUC primers #1233 and #1224 (NEB) using an ABI 373 automated sequencing system according to the manufacturer's instructions. Miniprep DNA preparations of the constructs were used as templates. The ends of the 3.3 kb amplified product from the *BstYI* circularized template and the 0.9 kb amplified product from the *HaeIII* circularized template matched with the known DNA region 3' to both inverse PCR primers Pmel-IP6 and Pmel-IP7. The complete sequence was obtained by primer walking and sequencing. The 3.3 kb product contained 2.9 kb new DNA sequence and the 0.9 kb product contained 200 bp new DNA sequence. No obvious methylase motifs were observed in the new DNA sequence.

[0074] 11. Amplifying and cloning DNA 3' to the endonuclease gene: Two consecutive inverse PCR amplification experiments were performed to obtain DNA information 3' to the endonuclease gene.

A. Inverse PCR amplification #1: Template preparation for inverse PCR amplification: 3 ug of genomic *Pseudomonas mendocina* DNA was digested with 100 U of *AatII* restriction endonuclease in 1X NEBuffer #4 (20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT, pH 7.9) in 100 ul reaction volume for 1 hour at 37°C. The restriction digestion mixture was extracted with one volume of equilibrated phenol:chloroform (50:50, v/v) and the aqueous phase was recovered and extracted with one volume of chloroform two times. The DNA was precipitated by the addition of 1/10th volume of 5 M NaCl and 1 volume of 2-propanol and then washed with 70% ice cold ethanol. The DNA was resuspended in 50 ul of 1XTE. 10 ul of this digested *Pseudomonas mendocina* DNA (approximately 0.5 ug) was circularized in 500 ul 1X ligase buffer using 2000 U of T4 DNA ligase at 16°C overnight. Circularized *HinfI*, *MscI*, *MseI*, *SacI*, *StuI* and *Tsp509I* digested *Pseudomonas mendocina* DNA were prepared in the same manner. These circularized DNA as well as the *BsaHI* digested, circularized DNA from section 5 were used as templates for subsequent inverse PCR reactions.

Primers Pmel-IP8 and Pmel-IP9 of sequences shown below were used to amplify DNA 3' to the endonuclease

gene. Both primers contain a *Pst*I sites to facilitate cloning.

5 Primer Pmel-IP8

5'-pGGTCTGCAGTCGGGCAGAACGTGATATCGA -3'

10

(SEQ ID NO:18)

15 Primer Pmel-IP9

5'-pGTTCTGCAGGCCATTGGCACCCCTG-3'

20

(SEQ ID NO:19)

In a reaction that was successful in amplifying the product, a reaction mix was made by combining:

25 10 ul of 10X Vent® reaction buffer (containing 2 mM Mg++ final concentration)

6 ul of 4 mM dNTP solution

5 ul of phosphorylated primer Pmel-IP8

5 ul of phosphorylated primer Pmel-IP9

12 ul of circularized DNA template (approximately 25 ng)

58 ul dH₂O

30

2 ul (4 units) of Vent® Exo- polymerase NEB#257

The PCR amplification conditions were: 95°C for 2 minutes for one cycle, followed by 5 cycles of 95°C for 20 seconds, 52°C for 30 seconds and 72°C for 2.5 minutes, followed by 20 cycles of 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 2.5 minutes. 15 ul of the PCR reaction was analyzed by electrophoresis on a 0.8 % agarose gel.

Among some non-specific amplified background DNA, a 1.1 kb product was observed in the *Aat*II circular template PCR reaction, a 1.4 kb product was observed in the *Msc*I circular template PCR reaction and a 0.7 kb product was observed in the *Bsa*HI circular template PCR reaction. These products were gel purified and resuspended in 20 ul of 1XTE. 2 ul of gel purified *Aat*II product was ligated to 100 ng of the vector pUC19 previously cleaved with *Sma*I, dephosphorylated with CIP and agarose gel purified, using 400 U of T4 DNA ligase in 20 ul final volume of 1X T4 DNA ligase buffer at 16°C for 2 hours. The gel purified *Msc*I and *Bsa*HI products were similarly ligated into pUC19 as described above. 10 ul of each ligation mixture was transformed into *E.coli* strain ER2267 and plated on L-broth plates containing 100 ug/ml ampicillin 40 ug/ml Xgal and 50 ug/ml IPTG for individual colonies. White colonies were individually picked to inoculate 10 ml of L-broth containing 100 ug/ml ampicillin and were allowed to propagate at 37°C overnight with agitation and aeration. Clones of the desired construct were identified by performing minipreps, digesting the purified DNA and analyzing it by agarose gel electrophoresis. The amplified products in these clones were sequenced using M13/pUC primers #1233 and #1224 (NEB). The newly obtained DNA sequence was used to design primers Pmel-IP10 and Pmel-IP11 shown below in a subsequent inverse PCR amplification to obtain more DNA sequence information 3' to this region.

50

Pmel-IP10

55 5'-phoshoto-GTTCTGCAGCCGTTCGATGGGCCTCTC-3'

(SEQ ID NO:20)

Pmel-IP11

5

5'- phospho-GTTCTGCAGAATGAGCTGCCAGTTG-3'
 10 (SEQ ID NO:21)

15 B. Inverse PCR amplification #2: *Hae*II digested and circularized *Pseudomonas mendocina* DNA (section 5) was used as template in this inverse PCR amplification with primers Pmel-IP10 and Pmel-IP11. In a reaction that was successful in amplifying the product, a reaction mix was made by combining:

20 10 ul of 10X Vent® reaction buffer (containing 2 mM Mg++ final concentration)
 6 ul of 4 mM dNTP solution
 5 ul of phosphorylated primer Pmel-IP10
 20 5 ul of phosphorylated primer Pmel-IP11
 12 ul of circularized DNA template (approximately 25 ng)
 58 ul dH₂O
 2 ul (4 units) of Vent® Exo- polymerase NEB#257

25 [0075] The PCR amplification conditions were: 95°C for 2 minutes for one cycle, followed by 5 cycles of 95°C for 20 seconds, 60°C for 30 seconds and 72°C for 2.5 minutes, followed by 20 cycles of 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 2.5 minutes. 15 ul of the PCR reaction was analyzed by electrophoresis on a 0.8 % agarose gel. A 0.7 kb amplified product was obtained and cloned into pUC19 vector previously cleaved by *Sma*I, dephosphorylated with CIP and agarose gel purified. Minipreps were performed to identify plasmid clones carrying the desired inserts. The 0.7 kb product was sequenced using M13/pUC primers #1233 and #1224 (NEB). Approximately 1.57 kb DNA sequence was obtained 3' to the endonuclease gene and no obvious methylase motifs could be found from the six frame amino acid translation of this DNA.

30 [0076] 12. Expressing *Dra*I methylase in vector pNEB193 to determine if DNA modified by *Dra*I methylase can be protected from *Pmel* restriction digestion: A *Dra*I methylase producing clone, pDraIRM9.7-G2, containing the *Dra*I methylase gene on a 5.5 kb insert in vector pBR322 (Jack Benner, New England Biolabs, Inc.) was partially digested with *Sau*3AI as follows: 7 ug of pDraIRM9.7-G2 DNA was diluted into 350 ul of 1X *Sau*3AI buffer (100 mM NaCl, 10 mM Bis Tris Propane-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.0). This mixture was used to prepare a serial dilution of *Sau*3AI restriction endonuclease from 1 unit/ug DNA to 1/64 unit/ug DNA by a factor of two each dilution in a final volume of 50 ul. These reactions were incubated at 37 °C for 15 minutes and the *Sau*3AI restriction endonuclease was subsequently heat-killed at 68°C for 20 minutes. 0.5 ug of the digested DNA produced from digestion of 1/8 unit of *Sau*3AI per ug of DNA was used to ligate to 200 ug of pNEB193 vector previously cleaved with *Bam*H I and dephosphorylated with CIP in 40 ul 1X T4 DNA ligase buffer containing 800 U of T4 DNA ligase for 16 hours at 16°C. The ligation mixture was transformed into 200 ul of *E.coli* strain ER2426 competent cells and plated on L-broth plates containing 100 ug/ml ampicillin. Approximately 10,000 colonies were obtained, and these were scraped into 6 ml of 10 mM Tris, 10 mM MgCl₂, pH 7.5. 2 ml of this pool was inoculated into 100 ml of L-broth containing 100 ug/ml ampicillin and allowed to grow at 37°C with shaking for 8 hours. Plasmids were isolated using a 10X scale-up of the miniprep procedure as described in section 5 above. The miniprep DNA was resuspended into 500 ul of 1XTE and 2 ul was digested with 40 units of *Dra*I in 50 ul of 1X NEBuffer 4 at 37°C for 2 hours. 10 ul of the digestion mixture was transformed into 50 ul of *E.coli* strain ER 2426 competent cells and plated on L-broth plates containing 100 ug/ml ampicillin. Plasmids from 9 individual transformants were isolated by miniprep procedure and digested with *Dra*I restriction endonuclease. Eight clones were fully protected from *Dra*I digestion. These were subsequently digested with *Pmel* restriction endonuclease and were fully protected from *Pmel* restriction digestion as well.

45 [0077] 13. Overexpressing the *Pmel* endonuclease:

50 [0078] A. Cloning the *Dra*I methylase on a compatible vector: The *Dra*I methylase gene was expressed by inserting the gene into the *Bam*H I site of pACYC184. To accomplish this, 0.5 ug of the *Sau*3AI partially digested pDraIRM9.7-G2 DNA prepared in section 12 was ligated into vector pACYAC184, previously cleaved with *Bam*H I and dephosphorylated, in 40 ul of 1X T4 DNA ligase buffer containing 800 U of T4 DNA ligase for 2 hours at 16°C. The ligation mixture was transformed into 200 ul of *E.coli* strain ER2426 competent cells and plated on L-broth plates containing 25 ug/ml

chloramphenicol. Approximately 1,600 colonies were obtained and these were scraped into 4 ml of 10 mM Tris, 10 mM MgCl₂, pH 7.5. 1 ml of this pool was inoculated into 75 ml of L-broth containing 25 µg/ml chloramphenicol and allowed to grow at 37°C with shaking for 8 hours. Plasmids were isolated using a 10X scale-up of the miniprep procedure as described in section 5 above. The miniprep DNA was resuspended into 500 µl of 1XTE and centrifuged at 14,000 rpm for 5 minutes. 336 µl of the cleared supernatant was mixed with 64 µl of 5 M NaCl and 400 µl of 13% PEG 8000 and incubated on ice for 1 hour. The DNA was spun down at 14,000 rpm at 4°C for 5 minutes, washed with ice cold 70% ethanol and air dried. The DNA pellet was resuspended in 500 µl of 1XTE and 5 µl of it was digested with 60 units of *Dra*I in 50 µl of 1X NEBuffer 4 at 37°C for 1 hour. 5 µl of this *Dra*I digested DNA was transformed into 50 µl of ER2426 competent cells and plated on L-broth plates containing 25 µg/ml chloramphenicol to obtain individual transformants.

10 Plasmids from 14 individual transformants were isolated by miniprep procedure and digested with *Dra*I restriction endonuclease, and of these twelve clones were fully protected from *Dra*I digestion. Further restriction analysis showed that two clones, pACYC184DraIM10 and pACYC184DraIM13, were identical and contained the smallest insert fragment of approximately 3kb. Clone pACYC184DraIM10 was used for *Pmel* endonuclease expression.

15 [0079] B. Endonuclease cloning: The restriction endonuclease gene was expressed by inserting the gene into an expression vector, pRRS, directly downstream of a strong inducible promoter (*P*lacUV5) and a strongly recognized ribosome binding site. To accomplish this, two oligonucleotide primers were made utilizing the DNA sequence data. The forward oligonucleotide primer contained a *Pst*I site to facilitate cloning, a stop codon in frame with the *lacZ* gene to terminate translation of the *lacZ* protein, a strongly recognized ribosome binding site, seven nucleotide spacer between the rbs and the ATG start codon of the *Pmel* endonuclease gene and 24 nucleotides complementary to *Pseudomonas mendocina* DNA for hybridization:

20

Primer PmelRexp1:

25 5'- GAGACTGCAGGAGGTATTATGACCACAACTCCCCCTCA
GAC-3' (SEQ ID NO:22)

30 [0080] The reverse primer was designed to hybridize to *Pseudomonas mendocina* DNA 84 bp 3' to the 3' end of the *Pmel* endonuclease gene. It contained a *Bgl*II site and a *Sac*I restriction site to facilitate cloning and 21 nucleotides complementary to *Pseudomonas mendocina* DNA for hybridization:

35 **Primer PmelRexp2:**

40 5'-CAAGAGATCTAGTCGACCGGTGTTAGCAACCCGATAC-3'
(SEQ ID NO:23)

These two primers were used to amplify the *Pmel* endonuclease gene from *Pseudomonas mendocina* genomic DNA by combining:

45 10 µl 10X Vent® reaction buffer
6 µl of 4 mM dNTPs
2 µl (400 ng) *Pseudomonas mendocina* genomic DNA
5 µl (10 µM stock) primer PmelRexp1
5 µl (10 µM stock) primer PmelRexp2
50 4 µl of 100 mM MgSO₄
66 µl dH₂O
0.6 µl (1.2 units) Vent® polymerase (2 unit/µl stock)

and amplifying at 95°C for 3 minutes for 1 cycle, followed by 4 cycles of 95°C for 30 seconds, 60°C for 20 seconds, 55 72°C for 50 seconds, followed by 20 cycles of 95°C for 30 seconds, 65°C for 20 seconds and 72°C for 50 seconds. The amplification product of approximately 850 bp was gel purified, cleaved with *Bgl*II and *Pst*I, phenol-chloroform extracted, precipitated, resuspended in TE and ligated into pRRS vector previously cleaved with *Pst*I and *Bam*H I and gel purified. The ligation reaction was transformed into *E. coli* strain ER2426 carrying the *Dra*I methylase gene construct

pACYC184DraI M10 and plated on L-broth plates containing 25 ug/ml chloramphenicol and 100 ug/ml of ampicillin for individual transformants. Seven individual transformants were inoculated into 10 ml of L-broth containing 25 ug/ml chloramphenicol and 100 ug/ml ampicillin, grown at 37°C with shaking to mid-log phase and induced with 0.5 mM IPTG for 16 hours. The cell pellets were spun down at 5000 rpm for 5 minutes at 4°C, resuspended in 1.5 ml of 20 mM Tris, 5

0.1 mM EDTA, 1 mM DTT, 50 mM NaCl, pH 7.5 and sonicated. The extracts were used to digest lambda DNA in 50 ul 1X NEBuffer #2 (10 mM Tris, 10 mM MgCl₂, 50 mM NaCl, 1mM DTT, pH7.9) at 37°C for 30 minutes. All clones expressed *Pmel* endonuclease activity. One of these clones, designated as pRRSPmelR1/pACYC184DraI M10; was selected for producing the *Pmel* endonuclease and given the strain designation of NEB #1081. A titration of the *Pmel* restriction endonuclease activity produced from crude extracts of NEB #1081 is shown in Figure 1. The enzyme titer 10 was approximately 1. X 10⁷ units/g of cells.

[0081] 14. The *Pmel* restriction endonuclease may be produced from NEB #1081 by propagation to mid-log phase in a fermenter containing L-broth medium with ampicillin (100 ug/ml) and chloramphenicol (25 ug/ml). The culture is induced by the addition of IPTG to a final concentration of 0.3 mM and allowed to continue growing for 16 hours. The cells are harvested by centrifugation and may be stored at -70°C or used immediately.

15 [0082] 15. Purification of the *Pmel* restriction endonuclease from NEB #1081 can be accomplished by a combination of standard protein purification techniques, such as affinity-chromatography or ion-exchange chromatography, as outlined in step 2 above. The *Pmel* restriction endonuclease obtained from this purification is substantially pure and free of non-specific endonuclease and exonuclease contamination.

20 [0083] A sample of the *E. coli* containing both pRRSPmelR1 and pACYC184DraI M10 (NEB#1081) has been deposited under the terms and conditions of the Budapest Treaty with the American Type Culture Collection on November 25, 1997 and received ATCC Accession Number 98596.

25

30

35

40

45

50

55

Annex to the description

[0084]

5

SEQUENCE LISTING

(1) GENERAL INFORMATION:

10

(i) APPLICANT:

- (A) NAME: New England Biolabs, Inc
- (B) STREET: 32 Tozer Road
- (C) CITY: Beverly
- 15 (D) STATE: Massachusetts
- (E) COUNTRY: US
- (F) POSTAL CODE (ZIP): 01915

15

(ii) TITLE OF INVENTION: Method for cloning and Producing the Pmel
20 Restriction Endonuclease

20

(iii) NUMBER OF SEQUENCES: 23

25

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

30

(2) INFORMATION FOR SEQ ID NO: 1:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

45

Met Thr Thr Asn Ser Pro
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

50

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

5
Met Ile Asp Glu Cys
1 5

(2) INFORMATION FOR SEQ ID NO: 3: _____

10
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15
(ii) MOLECULE TYPE: protein

20
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Asp Val Gly Met Ile Asp
1 5

25
(2) INFORMATION FOR SEQ ID NO: 4:

25
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

35
(ii) MOLECULE TYPE: protein

35
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

40
Met Thr Thr Asn Ser Pro Ser Asp Val Gly Met Ile Asp Glu Cys Leu
1 5 10 15
Ser Ile Val Xaa Thr Xaa Leu Ala
20

45
(2) INFORMATION FOR SEQ ID NO: 5:

45
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
50 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

55
(ii) MOLECULE TYPE: DNA (genomic)

55
(ix) FEATURE:

EP 0 931 835 A2

(A) NAME/KEY: Modified-site
(B) LOCATION:15
(D) OTHER INFORMATION:/product= "N=G, A, C OR T(U)"

5

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:18
(D) OTHER INFORMATION:/product= "N= G, A, C OR T(U)"

10

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:21
(D) OTHER INFORMATION:/product= "Y = C OR T(U)"

15

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:24
(D) OTHER INFORMATION:/product= "N = G,A,C OR T(U)"

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

25

Gly Thr Thr Gly Gly Ala Thr Cys Cys Ala Thr Gly Ala Cys Asn Ala
1 5 10 15
Cys Asn Ala Ala Tyr Thr Cys Asn Cys Cys
20 25

(2) INFORMATION FOR SEQ ID NO: 6:

30

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

40

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:15
(D) OTHER INFORMATION:/product= "N = G, A, C OR T(U)"

45

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:18
(D) OTHER INFORMATION:/product= "N = G, A, C OR T(U)"

50

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:21
(D) OTHER INFORMATION:/product= "Y = C OR T(U)"

55

(ix) FEATURE:
(A) NAME/KEY: Modified-site

(B) LOCATION:24
(D) OTHER INFORMATION:/product= "Y = C OR T(U)"

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Gly Thr Thr Gly Gly Ala Thr Cys Cys Ala Thr Gly Ala Cys Asn Ala
1 5 10 15
Cys Asn Ala Ala Tyr Ala Gly Tyr Cys Cys
20 25

10

(2) INFORMATION FOR SEQ ID NO: 7:

15

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

25

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:10
(D) OTHER INFORMATION:/product= "R = A OR G"

30

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:13
(D) OTHER INFORMATION:/product= "Y = C OR T(U)"

35

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:16
(D) OTHER INFORMATION:/product= "R = A OR G"

40

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION:19
(D) OTHER INFORMATION:/product= "D = G OR A OR T(U)"

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

50

Gly Thr Thr Cys Thr Gly Cys Ala Gly Arg Cys Ala Tyr Thr Cys Arg
1 5 10 15
Thr Cys Asp Ala Thr Cys Ala Thr
20

(2) INFORMATION FOR SEQ ID NO: 8:

55

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: protein

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

15 Asp Val Gly Met Ile Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 9:

20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Thr Asn Ser Pro Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 10:

40 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

50 Gly Thr Thr Gly Gly Ala Thr Cys Cys Gly Ala Cys Gly Thr Cys Gly
1 5 10 15

Gly Cys Ala Thr Gly Ala Thr Cys Gly Ala Cys Gly
20 25

55 (2) INFORMATION FOR SEQ ID NO: 11:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: RNA (genomic)

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

15 Gly Thr Thr Cys Thr Gly Cys Ala Gly Cys Thr Gly Ala Gly Gly Gly
1 5 10 15
Gly Cys Thr Gly Thr Thr Cys Gly Thr Thr Gly
20 25

20 (2) INFORMATION FOR SEQ ID NO: 12:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA (genomic)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

35 Gly Thr Thr Cys Thr Gly Cys Ala Gly Cys Thr Gly Ala Gly Gly Gly
1 5 10 15
Gly Cys Thr Gly Thr Thr Cys Gly Thr Thr Gly
20 25

40 (2) INFORMATION FOR SEQ ID NO: 13:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: protein

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

55 Val Gly Met Ile Asp

1 5

(2) INFORMATION FOR SEQ ID NO: 14:

5

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Gly Thr Thr Gly Gly Ala Thr Cys Cys Gly Thr Cys Gly Gly Cys Ala
20 1 5 10 15

Thr Gly Ala Thr Cys Gly Ala Cys Gly Ala
20 25

25 (2) INFORMATION FOR SEQ ID NO: 15:

30

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Cys Ala Thr Gly Gly Ala Thr Cys Cys Gly Ala Cys Gly Thr Cys Thr
40 1 5 10 15

Gly Ala Gly Gly Gly
20

45 (2) INFORMATION FOR SEQ ID NO: 16:

50

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Gly Thr Thr Cys Thr Gly Cys Ala Gly Cys Thr Cys Gly Thr Thr Gly
1 5 10 15
Thr Cys Thr Thr Cys Thr Thr Cys Thr Gly Cys
20 25

10 (2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (genomic)

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Ala Thr Cys Cys Thr Gly Cys Ala Gly Gly Cys Gly Ala Cys Gly Thr
25 1 5 10 15
Thr Cys Gly Gly Ala Cys Gly Ala Thr Gly
20 25

30 (2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Gly Gly Thr Cys Thr Gly Cys Ala Gly Thr Cys Gly Gly Gly Cys Ala
45 1 5 10 15
Gly Ala Ala Cys Gly Thr Gly Ala Thr Ala Thr Thr Cys Gly Ala
20 25 30

50 (2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

10

Gly Thr Thr Cys Thr Gly Cys Ala Gly Gly Cys Cys Ala Thr Thr Thr
1 5 10 15

15

Gly Gly Cys Ala Cys Cys Cys Thr Gly
20 25

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 29 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

30

Gly Thr Thr Cys Thr Gly Cys Ala Gly Cys Cys Gly Thr Thr Thr Cys
1 5 10 15

35

Gly Ala Thr Gly Gly Cys Cys Thr Thr Cys Thr Cys
20 25

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 27 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

50

Gly Thr Thr Cys Thr Gly Cys Ala Gly Ala Ala Thr Gly Ala Gly Cys
1 5 10 15

55

Thr Gly Cys Gly Cys Cys Ala Gly Thr Thr Gly
20 25

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: DNA (genomic)

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Gly Ala Gly Ala Cys Thr Gly Cys Ala Gly Gly Ala Gly Thr Ala
1 5 10 15

20 Ala Thr Thr Cys Ala Thr Ala Thr Gly Ala Cys Cys Ala Cys Ala Ala
20 25 30

Ala Cys Thr Cys Cys Cys Cys Cys Thr Cys Ala Gly Ala Cys
35 40 45

25 (2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

40 Cys Ala Ala Gly Ala Gly Ala Thr Cys Thr Ala Gly Thr Cys Gly Ala
1 5 10 15

Cys Cys Gly Gly Thr Gly Thr Thr Ala Gly Cys Ala Ala Cys Cys Cys
20 25 30

45 Gly Ala Thr Ala Cys
35

50 Claims

1. Isolated DNA coding for the *Pmel* restriction endonuclease, wherein the isolated DNA is obtainable from *Pseudomonas mendocina*,
- 55 2. A recombinant DNA vector comprising a vector into which a DNA segment coding for the *Pmel* restriction endonuclease has been inserted.
3. Isolated DNA coding for the *Pmel* restriction endonuclease and methylase, wherein the isolated DNA is obtainable

EP 0 931 835 A2

from *Pseudomonas mendocina* ATCC No. 98596.

4. A cloning vector which comprises the isolated DNA of claim 3.
5. The cloning vector of claim 4, wherein the cloning vector comprises both pRRSPmelR1 and pACYC184DraIM10.
6. A host cell transformed by the cloning vector of claim 2, 4 or 5.
7. A method of producing a *Pmel* restriction endonuclease comprising culturing a host cell transformed with the vector of claim 2, 4 or 5 under conditions for expression of said endonuclease.
10

15

20

25

30

35

40

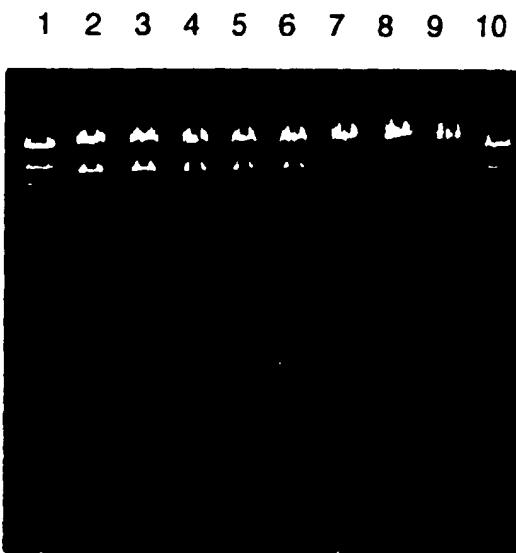
45

50

55

EP 0 931 835 A2

PmeI RESTRICTION ACTIVITY FROM THE E.coli STRAIN NEB #1081





(19)

Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) EP 0 931 835 A3

(12)

EUROPEAN PATENT APPLICATION

(88) Date of publication A3:
12.04.2000 Bulletin 2000/15

(51) Int Cl.7: C12N 15/55, C12N 15/54,
C12N 9/22

(43) Date of publication A2:
28.07.1999 Bulletin 1999/30

(21) Application number: 98309629.8

(22) Date of filing: 24.11.1998

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE

Designated Extension States:
AL LT LV MK RO SI

(30) Priority: 24.11.1997 US 976703

(71) Applicant: NEW ENGLAND BIOLABS, INC.
Beverly Massachusetts 01915 (US)

(72) Inventors:

- Chang, Zhiyuh
New Rochelle, NY 10805 (US)
- Morgan, Richard D.
Middleton, MA 01949 (US)

(74) Representative: Davies, Jonathan Mark
Reddie & Grose
16 Theobalds Road
London WC1X 8PL (GB)

(54) Method for cloning and producing the *Pmel* restriction endonuclease

(57) The present invention relates to recombinant DNA which encodes the *Pmel* restriction endonuclease

and modification methylase, and production of *Pmel* restriction endonuclease from the recombinant DNA.

EP 0 931 835 A3



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 98 30 9629

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | |
|---|--|--|--|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.) |
| X | EP 0 517 111 A (NEW ENGLAND BIOLABS INC) 9 December 1992 (1992-12-09) | 1,2,6,7 | C12N15/55 C12N15/54 C12N9/22 |
| Y | * abstract * * column 4, line 37 - column 5, line 40 * * column 3, line 20 - line 56 * * claim 6 * | 3-7 | |
| Y | EP 0 712 933 A (NEW ENGLAND BIOLABS INC) 22 May 1996 (1996-05-22) | 3-7 | |
| A | * example 1 * * page 12, line 58 - page 13, line 2 * | 1,2 | |
| Y | EP 0 778 346 A (NEW ENGLAND BIOLABS INC) 11 June 1997 (1997-06-11) | 5-7 | |
| | * page 6, line 26-43 * | | |
| A,D | LUNNEN K D ET AL: "CLONING TYPE-II RESTRICTION AND MODIFICATION GENES" GENE, NL, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 74, 1 January 1988 (1988-01-01), pages 25-32, XP002033330 ISSN: 0378-1119 * table I * | 1-7 | TECHNICAL FIELDS SEARCHED (Int.Cl.) C12N |
| The present search report has been drawn up for all claims | | | |
| Place of search BERLIN | | Date of completion of the search 18 February 2000 | Examiner ALCONADA RODRIG., A |
| CATEGORY OF CITED DOCUMENTS | | T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document | |
| X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document | | | |

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 98 30 9629

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EPO file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

18-02-2000

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---------------------|----------------------------|------------|---------------------|
| EP 0517111 A | 09-12-1992 | US | 5196330 A | 23-03-1993 |
| | | DE | 69201012 D | 09-02-1995 |
| | | DE | 69201012 T | 29-06-1995 |
| | | DE | 517111 T | 25-11-1993 |
| | | JP | 6098768 A | 12-04-1994 |
| EP 0712933 A | 22-05-1996 | US | 5543308 A | 06-08-1996 |
| | | JP | 8224088 A | 03-09-1996 |
| EP 0778346 A | 11-06-1997 | US | 5721126 A | 24-02-1998 |
| | | JP | 9275989 A | 28-10-1997 |